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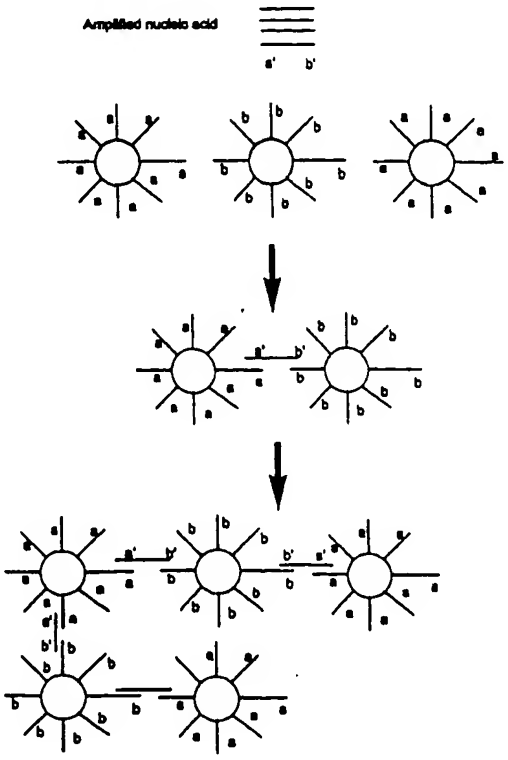
(57) Abstract

Methods and compositions for detecting a specified nucleic acid sequence in a sample by optically detecting formation of, or inhibition of formation of a hybridization complex as an indication of the presence of the specified nucleic acid sequence in a sample are disclosed.



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METHODS OF NUCLEIC ACID DETECTION

This application claims priority to U.S. Provisional Application Serial No. 60/063,969, filed October 31, 1997, under 35 U.S.C. §119(e).

FIELD OF THE INVENTION

The present invention relates to improved methods and compositions for detecting the presence of a target nucleic acid sequence in a sample, and specifically relates to visual detection of amplified nucleic acids using a particle agglutination assay.

BACKGROUND OF THE INVENTION

Nucleic acids (DNA and RNA) are basic components of biological systems. Detection, quantification, purification and other methods of characterizing nucleic acids are important tools in clinical diagnostic methods, development of therapeutic agents, agriculture and food processing, environmental detection of biological contaminants, basic research and the like. Although a variety of methods for detecting nucleic acids are known, because of the importance of nucleic acids in a wide variety of applications, there still exists a need for efficient and effective methods of nucleic acid detection. Moreover, because many of these applications require detection of specific base sequences or particular species of nucleic acid, there is a need for methods that detect specific nucleic acids in a sample.

Nucleic acids share common structural components (purine or pyrimidine bases, pentose sugars and phosphate-containing linkages), but the base sequence of a nucleic acid polymer generally determines the identity and function of a nucleic acid. Hybridization of complementary base sequences is generally used to detect individual nucleic acids and/or members of a related group of sequences in a sample. A base sequence characteristic of a particular nucleic acid or group of nucleic acids to be detected in an assay is often referred to as a "target nucleic acid sequence" or "target sequence." Target sequences include, for example, sequences characteristic of a microorganism, a virus, a plant, animal or human gene, and gene sequences characteristic of a pathogenic condition or indicating the presence of a pathogenic organism. A nucleic acid polymer containing a target sequence is often referred to as a target nucleic acid, where the target sequence is generally a subset of the base sequence of the polymer. A nucleic acid polymer may range in size from about 10 bases in length to many kilobases in length, but generally, smaller nucleic acids (e.g., up to a few hundred bases in length) are referred to as oligonucleotides, oligomers or polynucleotides.

A target sequence in a sample can be detected by hybridization methods that use nucleic acid probes to bind specifically to the target sequence, and not to other sequences in the sample. Hybridization is the process whereby a single strand of nucleic acid (RNA or DNA) binds to a complementary strand by hydrogen bonding or base pairing between a purine on one strand and a pyrimidine on the complementary strand (i.e., A:T and G:C pairing). Optimally, a probe binds only to a "specified sequence" that the assay is designed to detect specifically. The sequence to be detected may be present initially in the sample or may be produced from a template base sequence in the sample by a variety of methods. Specified sequences may include, for example, a target sequence, an amplified nucleic acid that includes the target sequence, a nucleic acid complementary to the target sequence or a "reporter group." A reporter group refers to a nucleic acid sequence that is produced in the presence of a target sequence, but which contains a base sequence different from the target sequence or its complement. For example, transcription-associated amplification of a target sequence may use a promoter-primer having a 3' primer sequence, a 5' promoter sequence, and a reporter group located between the primer and promoter to produce RNA transcripts incorporating the reporter group (as described in U.S. Pat. No. 5,399,491 to Kacian *et al.*).

Some methods of detecting a target sequence have used nucleic acid probes to hybridize to one or more target regions of a target nucleic acid. Multiple probes may be free in solution or bound to a fixed matrix or solid support. Methods of detecting nucleic acids using complementary nucleic acid probes, in which at least one probe is bound to a solid substrate, have been described in European Patent Application Nos. 0130523 and 0130515 by Dattagupta et al. Nucleic acids may be detected using a particle agglutination method in which nucleic acid sequences bound to inert particles are hybridized to complementary sequences in a sample, producing either agglutination of the inert particles or inhibiting agglutination of particles bearing complementary strands by binding competition (PCT Publication No. WO 87/05334, by Gefter et al.; Bains, W., 1998, *Clin. Chem.* 44(4): 876-878). Another agglutination hybridization method uses two labeled single-stranded polynucleotides that are complementary to the same or opposite strands of a target nucleic acid to form a double hybrid and/or a multi-hybrid of the probes and target nucleic acid (U.S. Pat. No. 5,288,609 to Englehardt et al.). Methods that combine polymerase chain reaction (PCR) amplification of target sequences and hybridization using matrix-affixed probes to detect single-stranded target sequences have been described by Brakel et al., European Pat. Publication No. 0435150. Hogan *et al.* (U.S. Pat. Nos. 5,451,503 and 5,424,413) disclose use of one or more probes to hybridize to a target nucleic acid, thus forming a detectable structure.

A variety of detection methods use solid phase-bound components in hybridization reactions. For example, sandwich hybridization methods that use both a soluble labeled nucleic acid probe and a solid phase-bound nucleic acid to bind to a target nucleic acid have been disclosed (U.S. Pat. Nos. 4,486,539 and 4,563,419 to Ranki et al.). Another known method uses oligonucleotide primers labeled with specific binding partner ligands to immobilize primers and primer extension products on a solid phase (European Pat. Publication No. 0370694 by Burdick et al.). U.S. Pat. No. 4,751,177 to Stabinsky describes a method that uses a mediator polynucleotide which hybridizes to a target sequence and to a polynucleotide fixed on a solid matrix, thereby producing a bound target to which a labeled probe is then hybridized, producing a signal with reduced background when the unbound labeled probe is washed away.

The invention disclosed herein is a hybridization detection method that uses a bridging oligonucleotide to enhance or inhibit formation of a hybridization complex which is visually detectable.

SUMMARY OF INVENTION

One aspect of the invention is a method of detecting a target nucleic acid in a sample. The method includes the steps of providing a sample containing multiple copies of a target sequence; mixing the multiple copies of the target sequence with a probe reagent comprising a first probe attached to a first detection group, and a second probe attached to a second detection group, thereby producing a hybridization mixture; incubating the hybridization mixture under conditions that favor hybridization of at least a portion of the first probe with a bridging molecule, and favor hybridization of at least a portion of the second probe with the bridging molecule, thereby producing a hybridization complex comprising the first probe and its attached first detection group, the second probe and its attached second detection group, and the bridging molecule; and optically detecting formation of the hybridization complex, thereby indicating that the target nucleic acid is present in the sample. In one embodiment, the multiple copies of the target sequence are present in the sample without further treatment of the sample, whereas in another embodiment, the multiple copies of the target sequence are produced by amplification of a target nucleic acid present in the sample. When amplification is used, the multiple copies are preferably produced by transcription-associated amplification of nucleic acid. In another embodiment, the bridging molecule is a target sequence present in the sample, an amplification product of a target nucleic acid present in the sample, a nucleic acid sequence capable of hybridizing to the amplification product of a target nucleic acid present in the sample, or a combination thereof. Preferably, the mixing and incubating steps are performed under essentially constant reaction conditions. In yet another embodiment, the bridging

molecule is an amplified nucleic acid, the first probe hybridizes, directly or indirectly, to a first amplified nucleic acid sequence, and the second probe hybridizes, directly or indirectly, to a second amplified nucleic acid sequence, thereby producing a hybridization complex comprising the first probe, the second probe, and the amplified nucleic acid. In one embodiment, the mixing step uses a first detection group that is an opaque particle and a second detection group that is a magnetically attractable particle, such that the hybridization complex formed in the incubating step is attractable by a magnetic field, thereby localizing the hybridization complex by application of a magnetic field during the detecting step. In another embodiment, the first and second detection groups used in the mixing step are made of acrylamide, and the incubating step produces a polyacrylamide gel phase that is optically detectable. Preferably, the detecting step uses visual detection of a change in the hybridization mixture without aid of instrumentation. In another preferred embodiment, in the mixing step, the first probe is an oligonucleotide having the sequence of SEQ ID NO:4 or SEQ ID NO:7, which is joined to an opaque particle using a 5' end or 3' end of the sequence, the second probe is an oligonucleotide having the sequence of SEQ ID NO:3 or SEQ ID NO:8, which is joined to a magnetically attractable particle using a 5' end or 3' end of the sequence, and the target sequence is characteristic of a *Mycobacterium complex* organism. In another embodiment, the first and second detection groups used in the mixing step have magnetic properties that are insufficient to make individual detection groups attractable by a magnetic field, and the incubating step produces a hybridization complex that is magnetically attractable by the magnetic field in the detecting step. Preferably, the first probe is covalently joined to the first detection group, and the second probe is covalently joined to the second detection group. In another embodiment, the mixing step also includes adding at least one linking molecule to the hybridization mixture, and the incubating step produces a hybridization complex that includes at least one linking molecule.

Another aspect of the invention is a method of detecting a target nucleic acid in a sample. This method includes the steps of providing a sample containing multiple copies of a target sequence; mixing the multiple copies of the target nucleic acid sequence with a probe reagent comprising a first probe attached to a first detection group, and a second probe attached to a second detection group, and a bridging molecule capable of hybridizing to both the first and second probes, thereby producing a hybridization mixture; incubating the hybridization mixture under conditions that favor hybridization of the first and second probes with the bridging molecule to produce a hybridization complex comprising the first probe and its attached first detection group linked to the second probe and its attached second detection group by hybridization with the bridging molecule; and optically detecting inhibition of formation of the hybridization complex by

competition hybridization between the target sequence and at least one of the first probe, the second probe or the bridging molecule.

Another aspect of the invention is a probe reagent composition that includes a first probe attached to a first detection group, and a second probe attached to a second detection group, wherein each of the probes includes a backbone made up of one or more sugar-phosphodiester type groups, one or more peptide nucleic acid groups, or a combination thereof, and independently selected sequences of bases which are adenine, guanine, cytosine, thymine, uracil or analogs thereof, joined to the backbone. In this probe reagent, each of the detection groups is made of a composition that, when aggregated, is capable of being optically detected as a change in characteristics of a solution containing the detection groups in a non-aggregated form. In preferred embodiments, the probe reagent also includes at least one linking molecule, which contains a base sequence capable of hybridizing to a selected sequence of bases contained in at least one probe. A particularly preferred embodiment includes a first probe having the sequence of SEQ ID NO:3 or SEQ ID NO:8, and a second probe having the sequence of SEQ ID NO:4 or SEQ ID NO:7.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates four examples of different hybridization complexes (one type for each line), where circles represent solid phase particles, lines represent oligonucleotides, and letters (a, b, c, d, a', b', c', d') represent base sequences, where complementary sequences are represented by the same letter without or with a prime symbol ('), e.g., the sequence "a" is complementary to the sequence "a'".

FIG. 2 illustrates the formation of an agglutination product, using the same format as in FIG. 1, where the first step (top) illustrates mixing of amplified nucleic acid strands containing sequences "a'" and "b'" with solid phase particles of two types, those with attached oligonucleotides containing sequence "a" or sequence "b"; a hybridization complex of an amplified nucleic acid strand and its complementary oligonucleotide sequence attached to the solid phase particles, thus bridging two different particles is shown below the upper vertical arrow; and, below the lower vertical arrow, an agglutination complex is shown, containing multiple particles, where each particle is attached to a different particle via a bridging nucleic acid that hybridizes to a complementary oligonucleotide sequence attached to a particle.

FIG. 3 illustrates the use of two different sets of probes (top line, labeled "A" and "B" within the circles) to detect the presence of different targets (labeled "NGO" and "CT" to refer to *Neisseria gonorrhoeae* nucleic acid and *Chlamydia trachomatis* nucleic acid, respectively); particles are

illustrated as in FIG. 1 except that numbers (1, 2, 3, 4) are used to illustrate binding sequences where sequences "1" and "2" specifically bind "NGO" (middle line) and sequences "3" and "4" specifically bind "CT" (bottom line) causing agglutination and resulting in a color change ("Red Drops Out" or "Blue Drops Out").

FIG. 4 illustrates the use of a ligase or polymerase-mediated primer extension reaction to form a more stable detectable hybridization complex (lower line, covalently linking particle "A" with particle "B") from a hybridization complex of the two particles (upper line) formed essentially as illustrated in FIG. 2.

FIG. 5 illustrates the use of a magnetically attractable detection group (particles labeled "A" and "B") to achieve solution clearing, where: the upper left illustrates a vessel containing a suspension of particles making the solution appear colored; the lower left illustrates formation of a hybridization complex in suspension, substantially as illustrated in FIG. 2,after bridging compounds are added to the vessel; and the lower right illustrates that, after application of a magnetic field to the vessel bottom, the hybridization complex is removed from suspension by magnetic attraction, resulting in solution clearing.

FIG. 6 illustrates the use of amplification oligonucleotides (bent solid lines) joined to detection groups (circles labeled "A" and "B") to amplify nucleic acid strands (extended solid or dashed lines, with arrow heads indicating polymerization) and to form hybridization complexes linking the detection groups, as shown in FIG. 6(C).

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes methods and compositions for detecting multiple copies of specified nucleic acid sequences by visually detecting at least one hybridization complex that includes the nucleic acid sequence and a detection group. Alternative embodiments of these methods detect the presence of multiple copies of specified nucleic acid sequences by visually detecting inhibition of hybridization complex formation. In these methods, nucleic acid is used as a bridging molecule joining together at least two probes to form a hybridization complex, or as a molecule which hybridizes to a bridging molecule, thereby inhibiting hybridization complex formation. The formation, or lack of formation, of a detectable hybridization complex indicates the presence of the specified nucleic acid sequence in a sample.

These methods are practiced in a number of preferred embodiments which allow visual detection, preferably without the use of instrumentation, of hybridization complexes or a change resulting from hybridization complex formation. In a "single-tube" format, amplification of nucleic acid, hybridization and complex detection are all performed in the same vessel using the same set of conditions. In one embodiment the hybridization complex contains a sufficient amount of magnetically attractable groups to allow the hybridization complex to be attracted by a magnetic field. In another embodiment, the hybridization complex cross-links probes coupled to polymeric material, resulting in a visually detectable change in the solution (e.g., changed viscosity). In one embodiment, nucleic acid amplification and detection uses the same oligonucleotides linked to detection groups. It will be appreciated by those skilled in the art that these formats, described above in terms of hybridization complex formation, can readily be adapted to assays in which inhibition of hybridization complex formation indicates the presence of the target nucleic acid in the sample tested.

As used herein, "amplified nucleic acid" refers to nucleic acid produced by any of a variety of known nucleic acid polymerization reactions, such as transcription-associated amplification, replicase-mediated amplification, or amplification that uses a polymerase chain reaction (PCR) or ligase chain reaction (LCR). These methods are well known in the art and have been previously described in detail (for transcription-associated amplification procedures see: U.S. Pat. No. 5,437,990 to Burg *et al.*; U.S. Pat. Nos. 5,399,491 and 5,554,516 to Kacian *et al.*; PCT Publication No. WO 93/22461 by Kacian *et al.*; PCT Publication Nos. WO 88/01302 and WO 88/10315 by Gingeras *et al.*; European Publication No. 0329822 A2 by Davey et al.; U.S. Pat. No. 5,130,238 to Malek *et al.*; PCT Publication No. WO 91/10746 by Urdea; PCT Publication No. WO 94/03472 by McDonough *et al.*; and PCT Publication No. WO 95/03430 by Ryder *et al.* ; for replicase-mediated

amplification methods see: Kramer *et al.*, U.S. Pat. No. 4,786,600, and Kramer and Lizardi, PCT Publication No. WO 90/14439; for PCR methods see: Mullis *et al.*, U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159, and in *Methods in Enzymology*, Volume 155, 1987, pp. 335-350; and for LCR methods see: Backman, European Pat. Publication No. 0320308). Amplified nucleic acid includes, for example, an amplified target sequence or its complement produced in an amplification reaction, or reporter group produced in an amplification reaction.

As used herein, a "hybridization complex" is made up of at least two probes hybridized directly, or indirectly, to a bridging molecule. A "bridging molecule" is a molecule that hybridizes directly or indirectly to at least two probes present in the hybridization complex. Multiple bridging molecules may link probes in a hybridization complex. Preferably, the bridging molecule is an amplified nucleic acid, a target nucleic acid, or a molecule that hybridizes to target nucleic acid or amplified nucleic acid. Amplified nucleic acid or multiple copies of target nucleic acid can function as bridging molecules, facilitating formation of a hybridization complex containing many probes and detection groups (e.g., from at least 4 to hundreds of probes and their associated detection groups). Alternatively, amplified nucleic acid or target nucleic acid can hybridize to the bridging molecule, inhibiting formation of a hybridization complex. Preferably, amplified nucleic acid acts as a bridging molecule.

Direct hybridization means that a first portion of the bridging molecule hybridizes to a first probe and a second portion of the bridging molecule hybridizes to a second probe via complementary base pairing. Indirect hybridization means that the hybridization complex includes one or more linking molecules. A "linking molecule" contains a base sequence that hybridizes to a probe, a bridging molecule, and/or another linking molecule via complementary base pairing. Linking molecules are optionally used to facilitate hybridization complex formation.

Probes, bridging molecules, and linking molecules are preferably nucleic acids (RNA or DNA) or analogs thereof capable of hybridizing to form a hybridization complex using complementary base pairing. Those skilled in the art will appreciate that a base sequence may participate in complementary base pairing even if the sequences are only partially complementary, contain base analogs (e.g., inosine) or contain abasic residues, so long as the sequence contains sufficient complementarity to allow hybridization under the selected assay conditions. Hybridization under selected assay conditions can readily be determined by those skilled in the art using standard testing or predicted using standard calculations (e.g., see Sambrook, J. et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2nd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) at pp. 1.90-1.91, 9.47-9.51 and 11.47-11.57).

Although nucleic acid probes, linking molecules and bridging molecules are preferred because of the simplicity of nucleic acid synthesis and hybridization, those skilled in the art will appreciate that these components could be any molecular form that is capable of specifically recognizing and binding to a single-stranded or double-stranded nucleic acid. For example, a probe or linking molecule may be an antibody that specifically recognizes DNA, RNA, or, more preferably a DNA:RNA duplex. Preferably, a monoclonal antibody that has these functional binding characteristics is used as a probe attached to a detection group or as a linking molecule.

Preferably, a probe is joined, directly or indirectly, to a detection group. A "detection group" refers to a group that produces, or aids in the production of, a detectable signal. The detection group is stably joined to a probe such that they do not dissociate during amplification and/or detection steps. A detection group that is more stable in the presence of target (e.g., becomes resistant to hydrolysis when associated with target sequence) is preferred. Numerous probes, that are the same or different, can be joined to a single detection group. The detection group can aid in the formation of a detectable signal by, for example, being a signal-generating label that produces, with or without additional treatment, a detectable signal. Preferred detection groups are particles that can produce a detectable signal without additional treatment.

A hybridization complex generally contains a first probe joined to a first detection group, and a second probe joined to a second detection group, both probes joined directly or indirectly via a bridging molecule, and/or linking molecules. The "first" and "second" detection groups may be identical or different compositions. Optionally, a hybridization complex may be stabilized by covalently joining first probes to second probes, either directly (first probe to second probe linkage) or indirectly (joining via sequences contributed from one or more linking molecules). For example, first and second probe sequences may be aligned by hybridization to a bridging molecule in a hybridization complex such that a ligase reaction will covalently link the ends of the two primers, thus stabilizing the complex even if the bridging molecule was subsequently removed. Similarly, first and second probe sequences may be aligned by hybridization to a bridging molecule in a hybridization complex such that a primer extension reaction beginning at one probe's end (serving as a primer) and using the bridging molecule as a template strand may join the first and second probes using via sequence complementary to a portion of the bridging molecule. Thus, an optional enzymatic reaction may result in a more stable hybridization complex.

One embodiment is a method that uses a single set of conditions to produce amplified nucleic acid when a target sequence is present in a sample, and hybridize the amplified nucleic acid to produce, or inhibit production of, a visually detectable hybridization complex that indicates that the

target sequence was present in the tested sample. The method involves exposing a sample to amplification conditions to produce amplified nucleic acid if the target sequence is present. The amplified nucleic acid participates in formation of, or inhibits formation of, a hybridization complex that includes a first probe, a second probe, and a bridging molecule. "Amplification conditions" refer to conditions compatible with nucleic acid polymerization and/or ligation using at least one enzyme to produce a nucleic acid strand complementary to a template strand. Such conditions are well known in the art and depend on the type of amplification method used, but generally include enzymes, amplification oligonucleotides, nucleoside triphosphate substrates, salts, buffers, and appropriate incubation temperature. "Amplification oligonucleotides" are oligonucleotides that hybridize to a target nucleic acid or its complement, and participate in an amplification reaction, generally as primers or promoter-primers.

In preferred embodiments, amplified nucleic acid that participates in the detection step contains the target sequence or its complement. In other embodiments, amplified nucleic acid that participates in the detection step is a sequence whose amplification is triggered by the presence of a target sequence resulting in (1) an amplified nucleic acid related to the target sequence or (2) an amplified nucleic acid not related to the target sequence. Amplified nucleic acid related to the target sequence uses the target sequence, or its complement, as a template. Amplified nucleic acid not related to the target sequence can be used as a reporter group and is produced using a template that is not the target sequence or the complement thereof.

In one embodiment, the presence of multiple copies of a specified base sequence in a sample is detected by visually detecting a hybridization complex that contains a sufficient amount of magnetically attractable detection groups to be attracted by a magnetic field. The multiple copies of the specified base sequence may be a target sequence initially present in the sample or may be produced by amplification. For example, it may be amplified nucleic acid containing the target sequence or its complement, or a reporter group. As described above, the hybridization complex contains at least two probes and a bridging molecule, and may optionally include one or more linking molecules. During the detecting step, a magnetic field is applied to a vessel containing the hybridization complex that includes a sufficient number of magnetically attractable detection groups to be attracted by the magnetic field and thus concentrated into a visually detectable signal. If an insufficient number of attractable detection groups are present, or no hybridization complex has formed, no visually detectable signal is produced in response to the magnetic field.

Another embodiment uses groups made up of a polymeric material (e.g., polyacrylamide, protein, polyacrylate, or albumin-coupled oligonucleotides) to detect the presence of a specified

base sequence in a sample. The detected sequence may be initially present and/or may be produced by amplification. Formation of a hybridization complex that includes the detected sequence cross-links the polymeric material resulting in a visually detectable phase change, such as a phase transition from a liquid to a gel.

One embodiment uses oligonucleotides joined to a detection group to serve as both primers for amplifying a target sequence and probes for detecting the amplified nucleic acid. These oligonucleotides are referred to as "primer/probes." Preferably, the method uses a transcription-associated amplification that employs a first primer/probe comprising a 5' promoter sequence and an extendable 3' end sequence that hybridizes to the target sequence, and a second primer/probe comprising an extendable 3' end sequence that hybridizes to the complement of the target sequence. An "extendable 3' end sequence" refers to a 3' sequence that hybridizes to a complementary sequence and primes a primer extension reaction catalyzed by a nucleic acid polymerase. A "5' promoter sequence" is a sequence located 5' of the 3' end, that binds an RNA polymerase and initiates an RNA polymerase reaction; the 5' promoter sequence is 5' of the 3' end and may, or may not, be located at the 5' end of the probe.

Transcription-associated amplification has been described in detail previously (e.g., see U.S. Pat. Nos. 5,399,491 and 5,554,516 to Kacian *et al.*; PCT Publication No. WO 93/22461 by Kacian *et al.*) and is well known to those skilled in the art. Briefly, transcription-associated amplification reaction conditions refer to conditions (salts, buffers, substrates, enzymes and temperature) for performing transcription-associated amplification in the presence of amplification oligonucleotides and target nucleic acid. The preferred method uses essentially constant reaction conditions (i.e., conditions that are not altered to denature or anneal nucleic acids) to amplify nucleic acid and detect the presence of amplified nucleic acid.

One advantage of the methods described herein is that the presence of a hybridization complex is visually detectable without the aid of instrumentation. Such methods can be used, for example, to detect the presence of pathogenic microbes without use of detection equipment. Because of this relatively simple method of detection, the methods are suitable for use in relatively unsophisticated laboratory settings such as mobile laboratories or field testing.

A variety of different probe configurations can be used to produce hybridization complexes of at least two probes hybridized directly or indirectly to a bridging molecule. Although FIG. 1 illustrates some examples of hybridization complexes formed using different probe configurations, other types of hybridization complexes are apparent from the descriptions provided herein.

FIG. 1(A) diagrams a complex formed between a first probe (containing sequence "a") joined to a first detection group (shown by the large circle), a second probe (containing sequence "b") joined to a second detection group (shown as a circle), and a bridging molecule (shown by the line labeled at the ends with "a'" and "b'"). The bridging molecule includes sequences "a'" and "b'" that are complementary to sequences "a" and "b".

FIG. 1(B) shows a similar hybridization complex that includes one probe containing sequence "a" linked to a detection group (circle), a second probe containing sequence "d" linked to a detection group (circle), and the presence of two linking molecules (one line labeled "a'" and "b'" and a second line labeled "c'" and "d'") to form a hybridization complex, in which the probes are hybridized indirectly to the bridging molecule (the line labeled "b" and "c"). The complementary sequences of these probes, linking molecules and bridging molecule are aligned as above (i.e., "a" is complementary to "a'" and so on).

FIG. 1(C) illustrates a multiple hybridization complex where multiple first probes (lines labeled "a") are joined to a first detection group (circle) and multiple second probes (lines labeled "b") are joined to a second detection group (circle). The hybridization complex is formed by bridging molecules (lines labeled "a'" and "b'") that hybridize to first probes and second probes by pairing of complementary sequences ("a'" with "a", and "b'" with "b").

FIG. 1(D) shows a multiple hybridization complex similar to that illustrated in FIG. 1(C) except that each detection group (circle) is joined to multiple first probes (lines labeled "a") and multiple second probes (lines labeled "b"). The hybridization complex is formed by bridging molecules as described for FIG. 1(C).

A sample that contains multiple copies of a target sequence will more readily produce hybridization complexes in which many detection groups are joined in a larger aggregate. Because many samples may not contain a sufficient number of copies of target sequences to form such aggregates, amplification of nucleic acid in the sample is preferred for visual detection of hybridization complexes. Furthermore, with increased numbers of copies, the kinetics of hybridization will be faster, allowing detection of the aggregates in a shorter amount of time. For example, for a reaction mixture of about 0.2 ml, the number of target sequences that produce an optically detectable change in the mixture would be in a range having a lower limit of about 100 fmoles to about 500 fmoles, more preferably having a lower limit of about 50 fmoles to about 125 fmoles, and even more preferably having a lower limit of about 10 fmoles to about 75 fmoles. Hybridization complex formation and detection has no upper limit of numbers of detectable target sequences, so long as the amount does not produce conditions (e.g., viscosity) that interfere with

hybridization.

FIGS. 2 to 5 illustrate some of the embodiments described herein in which hybridization complexes are formed and optically detected.

Referring to FIG. 2, one embodiment detects multiple copies of nucleic acid (lines labeled "a'" and "b'") by forming hybridization complexes that includes a first probe (line labeled "a") that is joined to a first detection group (circle) and a second probe (line labeled "b") that is joined to a second detection group (circle). As shown in FIG. 2, the multiple copies of nucleic acid to be detected can be amplified nucleic acid (top of FIG. 2) which is incubated with the probes described above. The first probe hybridizes to a region at the 5'-end of amplified nucleic acid (labeled "a'") and the second probe hybridizes at the 3'-end of the same amplified nucleic acid (labeled "b'") to form a hybridization complex joined by a bridging molecule, the amplified nucleic acid (shown below the upper vertical arrow). As hybridization or cross-linking continues, more hybridization complexes are formed (shown below the lower vertical arrow). A sufficient amount of cross-linking produces an agglutination product that precipitates out of solution and is visually detected by formation of a precipitate.

Insoluble detection groups can be used to facilitate detection of the precipitated agglutination product. For example, colored detection groups that remain suspended in solution in the absence of hybridization with multiple copies of the nucleic acid to be detected can be used to visually detect (1) clearing of the solution due to the agglutination product falling out of solution, and/or (2) a colored precipitate of the hybridization complex agglutination product. Preferably, visual detection does not require use of instrumentation to detect the presence of a hybridization complex.

FIG. 3 illustrates a specific example of the embodiment diagramed in FIG. 2. Here, two sets of probes attached to different colored detection groups are used to detect the presence of different organisms in a sample. Referring to the top of FIG. 3, the first probe set is targeted to *Neisseria gonorrhoeae* nucleic acid and contains a first probe (lines labeled "1") joined to a red latex particle as the detection group (circle labeled "A") and a second probe (lines labeled "2") also joined to a red latex particle detection group (circle labeled "A"). The second probe set is targeted to *Chlamydia trachomatis* nucleic acid and contains a third probe (lines labeled "3") joined to a blue latex particle detection group (circle labeled "B") and a fourth probe (lines labeled "4") also joined to a blue latex particle detection group (circle labeled "B").

When both sets of probes are in solution, the solution will be purple in color due to the combination of red and blue latex particles. The presence of multiple copies of nucleic acid capable of hybridizing to one or both of the probe sets (i.e., from one or both of the target organisms)

changes the appearance of the sample. In the presence of *Neisseria gonorrhoeae* nucleic acid copies (lines labeled "NGO"), hybridization complexes are formed, as described for FIG. 2, with the red latex particles, resulting in agglutination that causes the red particles to drop out of solution, but the blue particles remain suspended in solution. That is, the solution changes from purple to blue if *N. gonorrhoeae* nucleic acid is present in the sample. In the presence of *Chlamydia trachomatis* nucleic acid (lines labeled "CT"), hybridization complexes are formed with the blue latex particles, as described for FIG. 2, resulting in agglutination that causes the blue particles to drop out of solution, while the red particles remain suspended. That is, if *C. trachomatis* nucleic acid is present in the sample, the solution turns from purple to red. If both *N. gonorrhoeae* and *C. trachomatis* nucleic acids are present in the sample, both the red and the blue particles are precipitated out of solution resulting in clearing of the purple solution color. The nucleic acid initially present in a sample, such as a biological swab sample, can be amplified using any of a variety of methods to provide a sufficient amount of target nucleic acid to cause agglutination that results in precipitation of the products and a visually detectable change in the appearance of the solution.

FIG. 4 illustrates the optional use of a primer extension or ligase reaction to form a more stable hybridization complex. The top of FIG. 4 shows the first and second probes joined to different detection groups (circles labeled "A" and "B") that are linked non-covalently by hybridization to a bridging molecule as described above. The vertical arrow represents a ligase reaction and/or a polymerase-mediated primer extension reaction, resulting in a covalent linkage between the first and second probes, thus producing a more stable hybridization complex. It will be understood by those skilled in the art that similar covalent linkage could be achieved using ligation or primer extension reactions to join a series of molecules (e.g., linking molecules) for those hybridization complexes that include more that bridging molecules and probes.

FIG. 5 illustrates an example of the embodiment that uses a magnetic field to attract hybridization complexes that contain sufficient numbers of magnetically attractable particles. As shown in FIG. 5, upper left, the reaction vessel contains a mixture of particles suspended in solution. One set of particles have a first probe joined to a detection group with magnetic properties (circles labeled "A"). The second set of particles have a second probe joined to a colored detection group (circles labeled "B"), such as a colored latex bead. As shown in the upper left of FIG. 5, initially the detection groups are suspended in solution providing a colored solution. As shown in the lower left of FIG. 5, in the presence of multiple copies of a nucleic acid bridging molecule, the first probe hybridizes to a first region of the nucleic acid and the second probe hybridizes to a second region of the nucleic acid forming a hybridization complex as described above. The multiple copies of the

nucleic acid bridging molecule may be present initially in the sample tested or more be increased in number using any of a variety of nucleic amplification methods. As shown in the lower right of FIG. 5, an external magnetic field is then used to localize the hybridization complex containing the colored detection groups in a specific spatial location, such as the side or bottom of the reaction vessel. Formation of hybridization complexes followed by the application of a magnetic field results in solution clearing due to localization of the colored detection groups. The solution clearing is a visually detectable signal showing the presence of the target nucleic acid in the reaction mixture.

A similar methodology may use first and second detection groups which individually lack sufficient mass to be attracted by a magnetic field, but when cross-linked during hybridization exhibits sufficient paramagnetic properties. An aggregate, such as that shown in FIG. 5, lower left and lower right, illustrates this principal. A magnetic field can be applied at different time points during the assay, or can be continuously applied because the hybridization complex will only become attractable when sufficient numbers of magnetically attractable detection groups are present in the complex.

Another preferred embodiment which is not illustrated employs a polymer detection group (e.g., acrylamide) linked to probes and uses multiple copies of nucleic acid as the bridging molecules. The bridging molecules may be initially present in the sample or may be increased in number by nucleic amplification. Both the first probe and the second probe are joined to polymer detection groups. The formation of a hybridization complex cross-links the detection groups forming large polymeric complexes that are visually detectable by observing a physical change in the reaction solution. In the case of acrylamide detection groups, crosslinking in the hybridization complexes produces a visually detectable change in the solution from a liquid to a gel phase. Other polymer detection groups (e.g., plastics, such as polyacrylate, or proteins, such as albumin) can be similarly detected by a phase change resulting from cross-linking of the detection groups in hybridization complexes.

Preferably, multiple copies of bridging molecules are generated from nucleic acids in a sample using a nucleic acid amplification process. By amplifying nucleic acid present in a sample, large amounts of nucleic acid bridging molecules are produced, thereby increasing the number and/or size of hybridization complexes that are visually detectable. Different amplification techniques can be used to generate amplified nucleic acids, such as transcription-associated amplification, replicase-mediated amplification, a polymerase chain reaction (PCR) or a ligase chain reaction (LCR). These techniques are well known in the art and have been described in detail elsewhere (see references cited above).

Preferred amplification techniques are those performed under essentially constant reaction conditions, such as transcription-associated amplification, meaning that the reaction conditions (e.g., temperature) are not altered to denature or anneal nucleic acid. Preferably, essentially constant reaction conditions employ a temperature that does not change more than about 15°C, more preferably no more than about 5°C, and most preferably no more than about 1°C. Preferably, multiple rounds of amplification are carried out by polymerase reactions that use a product of one round of amplification as the starting material for another round of amplification. Carrying out multiple rounds of amplification using essentially constant reaction conditions provides for continuous generation of amplified nucleic acid and formation of hybridization complexes because the hybridization complexes are not disrupted during amplification.

Amplification oligonucleotides are nucleic acids used to produce multiple copies of the nucleic acid to be detected and the sequences chosen will depend on the amplification procedure and the nucleic acid being amplified. Those skilled in the art can readily design or select appropriate amplification oligonucleotides suitable for amplification of a target nucleic acid or a related sequence indicative of the presence of a target sequence in the sample. An amplification oligonucleotide may be analogous or complementary to a target nucleic acid and may include additional functional sequences such as a promoter sequence a promoter-primer.

A preferred amplification method is transcription-associated amplification which uses an RNA polymerase to produce multiple RNA transcripts from a nucleic acid template under essentially constant conditions. Various methods of transcription-associated amplification are well known in the art. A transcription-associated amplification procedure that uses RNase H activity is preferred (as previously described in detail in U.S. Pat. No. 5,399,491 to Kacian *et al.*, which uses RNase H activity present in a reverse transcriptase).

FIG. 6 illustrates a method that uses amplification oligonucleotides attached to detection groups to produce multiple copies of the nucleic acid to be detected visually in hybridization complexes. FIG. 6 includes steps A) to E) which show a transcription-associated amplification reaction in which hybridization complexes are formed during the amplification process. Referring to FIG. 6, the "+" and "-" designations at the right refer to strand orientations, where "+" indicates a strand in 3' to 5' orientation and "-" indicates a strand in 5' to 3' orientation. FIG. 6, step A) illustrates a first probe (shown as a bent solid line) attached to a first detection group (the circle labeled "A") hybridized to target nucleic acid (a "+" strand; upper solid line) and primer extension of the 3' end of the probe to form a "-" DNA strand (lower dashed line) that is complementary to the "+" target strand. The first probe is a template-complementary oligonucleotide that includes a 5' promoter sequence

(labeled "P") recognized by an RNA polymerase and a 3' sequence that hybridizes to the target nucleic acid. Formation of the "-" strand results in a duplex and a portion of the "-" strand becomes available for hybridization to a second probe by removing all, or a portion, of the "+" strand, such as by heat denaturation or enzymatic digestion with RNase H when the initial "+" strand is RNA.

Referring to FIG. 6, step B), a second probe is provided that includes an oligonucleotide (shown as a bent solid line) joined to a second detection group (circle labeled "B"). In step B), the second probe hybridizes to the "-" strand and primer extension of the second probe produces a "+" DNA strand (dashed upper line) complementary to the "-" strand (solid lower line). FIG. 6, step C) shows the resulting hybridization complex that contains a "+" strand joined to a "B" detection group and a "-" strand joined to a "A" detection group, and having a double-stranded DNA promoter sequence ("P") near one end. This hybridization complex can be used to produce additional hybridization complexes without being disrupted by using an RNA polymerase that recognizes the promoter sequence "P" to produce multiple copies of "-" sense RNA transcripts.

FIG. 6, step D) shows a "-" strand RNA transcript (solid line) produced from the duplex shown in step C). Then a second probe, as described in step B), that includes an oligonucleotide (shown as a bent solid line) joined to a second detection group (circle labeled "B") hybridizes to the RNA transcript and primer extension (shown as the dashed upper line) from the second probe forms an RNA:DNA duplex.

FIG. 6, step E) shows a region of the "+" strand of step D) that is hybridized to the first probe attached to particle "A", without disrupting the hybridization complex formed in step C). For example, RNAse H activity can digest the "-" RNA strand of an RNA:DNA duplex of step D), without disrupting the DNA:DNA duplex of step C). When the first probe attached to particle "A" is hybridized to the "+" strand, primer extension of the first probe (lower dashed line), and primer extension of the 3' end of the DNA "+" strand formed in step D) proceeds. The resulting hybridization complex is identical to that shown in step C). Multiple cycles of steps C) to E) produce multiple copies of the hybridization complex illustrated in step C), which are then visually detectable due to the number of complexes that link detection groups "A" and "B".

Formation of a hybridization complex, such as those described above, relies on base sequences contained in a first probe, a second probe, a bridging molecule, and optional linking molecules which allow these components to hydrogen bond. Hybridization between two complementary sequences depends on the reaction conditions (e.g., salt concentration, temperature, denaturing agents) and the degree of hydrogen bonding between the sequences, which is affected by the GC content and length of the complementary sequences, and the number

and location of mismatches, as is well known in the art (e.g., see Britten and Davidson, in *Nucleic Acid Hybridization: A Practical Approach*, pages 3-14, IRL Press, Washington (1985); and Sambrook *et al, Molecular Cloning, 2nd ed.*, (1989, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Chapter 11).

Hybridization complexes can be formed using nucleic acid components that are substantially complementary sequences or completely complementary sequences (i.e.,100% complementarity). "Substantially complementary sequences" have a lesser degree of complementarity but sufficient complementarity to allow specific hydrogen bonding between the two sequences. For the methods described herein, preferred substantially complementary sequences involve two nucleic acid sequences in which one molecule contains at least about 10, and more preferably at least about 15 adjacent bases, of which at least about 80%, and more preferably at least about 90% can hydrogen bond with a base sequence present on the second molecule, under the reaction conditions chosen.

Probes used in these methods hybridize specifically to the nucleic acid to be detected, or to one or more linking molecules, while not hybridizing to other nucleic acids in the sample. Those skilled in the art can readily determine useful probe sequences based on the T_m values of the expected probe:target or probe:linking molecule hybrid under the chosen reaction conditions. That is, for the chosen reaction conditions, the T_m of a probe:target (or probe:linking molecule) hybrid should be higher than the T_m of potential probe:non-target hybrids, such that formation of a probe:target hybrid is favored in the reaction conditions.

Hybridization between a probe sequence and a complementary sequence can be facilitated by using a "spacer" sequence that joins the hybridizable probe sequence to a detection group or particle, or is a separate molecule that joins the probe sequence to a detection group or particle. The spacer separates the detection group or particle from the hybridizable probe sequence, thereby reducing any adverse effect of the particle on hybridization between the probe sequence and a complementary sequence. Preferred spacers are nucleic acids of at least about 5 nucleotides, more preferably at least about 15 nucleotides, and more preferably about 15 to about 30 nucleotides in length. Similarly, the methods described herein may use helper probes to facilitate hybridization between complementary nucleic acids by decreasing the adverse effect on hybridization of secondary structure, as described in detail in U.S. Pat. No. 5,030,557 to Hogan *et al.*

As discussed above, the methods described herein have the advantage of providing a detectable signal, preferably a signal that does not require the use of instrumentation in the detection step. That is, simple visual inspection of the reaction is preferred to detect the changes resulting from hybridization complex formation.

A variety of changes in sample characteristics may be detected, based on physical characteristics of a hybridization complex detectable by optical means. Changes may be localized to a particular location within, for example, a sample tube, vessel or solution. More preferably, the presence of amplified nucleic acid is detected by visual means without the aid of instrumentation.

Hybridization complex formation concentrates detection groups within localized areas of a vessel or solution, relative to the otherwise random distribution of such groups. Localized increased concentration of detection groups allows visual detection of the hybridization complexes. While formation of simple hybridization complexes (such as shown in FIG. 1A) links two detection groups, the formation of hybridization complexes containing many detection groups effectively localizes or concentrates many detection groups and promotes precipitation of the hybridization complexes.

In one embodiment, detecting a change in sample appearance is aided by using a first probe joined to a first detection group and a second probe that contains a detection group used to localize a hybridization complex (e.g., a magnetically attractable particle) and/or that is bound to a solid matrix that is not free in solution. By localizing the position of the hybridization complex the detection group will be positioned in a particular location thereby facilitating its detection.

Although less preferred than visual detection of changes in sample appearance, instrumentation may be used to measure sample changes, thus facilitating detecting smaller amounts of hybridization complexes. For example, a spectrophotometer, Coulter Counter or near infra-red turbidimeter may be used to detect changes. Measurements of physical or chemical changes are well known in the art, e.g., measurement of changes in turbidity detected as light transmittance at 940 nm for latex suspensions or light absorbance at 585 nm to detect latex agglutination. Other optical methods of detecting sample changes that are well known in the art include, for example, microscopic detection of hybridization complexes; nephelometry to detect changes in light absorbance compared to a standard curve to generate a semi-quantitative result; quasi-elastic light scattering (also known as Photon Correlation Spectroscopy or Laser Doppler Assay) which uses reflected light from a laser-illuminated particle solution to distinguish between single particles and complexed particles; and angular anisotropy which measures light scatter through a test sample at two angles (e.g., 45^0 and 135^0), and determines the degree of agglutination using the ratio of forward/backward scattered light.

A signal generating label used as the detection group may also provide an optically detectable signal. Preferred signal detection labels are those that can be used under amplification conditions, e.g., fluorescent groups and radioisotopes. Fluorescent groups are preferred, particularly those that absorb light above 400 nm and emit light at wavelengths at least 10 nm higher

than the excitation wavelength. Particularly preferred fluorescent groups include porphyrins, 2-aminoaphthalene, p,p'-diaminobenzophenone imines, 1,2-benzophenazin, and quaternary phenanthridine salts. Radioisotopes, such as ^{3}H, ^{14}C, ^{32}P, and ^{35}S, are less preferred signal generating labels, some of which can produce a visually detectable signal upon exposure to X-ray film.

Detecting the presence of hybridization complexes containing a signal generating label is typically carried out by determining the presence of the label in a particular location, such as the bottom of a test tube using magnetically attractable particles. For example, at different time points in an assay the location where hybridization complexes are expected to concentrate can be irradiated and the fluorescence observed. An increase or decrease in the fluorescence at the location over two or more time points indicates a change in the amount of hybridization complexes. Because of the versatility of the present invention, various detection formats may be used such as irradiating the entire sample at a single time point and determining the level of signal observed compared to a standard in which no hybridization complexes can be formed.

To aid in visual or optical detection, a hybridization complex may be localized in a test sample using any of a variety of detection groups or by directly joining a probe to a solid matrix. Detection groups are generally particles that can be suspended in a solution whereas a solid matrix generally refers to a surface not suspended in solution, to which a probe can be attached. Detection groups and/or a solid matrix can be made of a variety of materials, such as latex, carbon, metals (e.g., colloidal gold), nitrocellulose, nylon, glass such as controlled pore glass, or plastics such as polystyrene, polypropylene, polyvinylchloride, and styrene divinylbenzene.
Detection groups also can be made from liposomes, agarose and polyacrylamide. Particulate detection groups preferably are of a size and shape that allows them to remain in suspension in a solution in the absence of hybridization complex formation. For example, the particles generally have a maximum dimension of between about 0.1μm and about 1.0μm, more preferably between about 0.3μm and about 0.9μm, and even more preferably between about 0.4μm and about 0.7μm.
Preferred particle detection groups are made of a colored and/or opaque material such as colored latex beads or colloidal gold, or include a metal with paramagnetic properties. Other readily available latex beads suitable for use in the present methods have fluorescent groups or dyes to aid in detection of hybridization complexes. Probe-linked particle detection groups are preferably initially free in solution or suspension.

Detection groups can be attached using standard techniques to a probe by a covalent linkage, chelation, ionic interactions, or through use of a binding partner set (i.e., ligand and hapten)

where one member is present on the detection group and the second member is present on the probe. A detection group is preferably attached to a probe by a covalent linkage, such as, for example, a non-nucleotide linkage. The first probe and second probes can each individually be attached to a detection group using the probe's 3' end, 5' end, or an internal location, or any combination thereof for individual probes on the detection group. For example, a first probe may be attached to a detection group using a 5' end, and a second probe may be attached to a detection group using an internal residue, or through its 3' or 5' end. Spacers are particularly useful when a detection group is attached internally to a probe, and when both the first and second probes are attached to a detection group by their 3' end or 5' end.

Preferred detection groups used to localize a hybridization complex contain a group that binds to a solid matrix or is magnetically attractable because of its paramagnetic properties. Additionally, particles not sufficiently magnetic alone, but paramagnetic in the aggregate, may also be used for hybridization complex localization. Factors affecting the ability of a particle or aggregate of particles to be magnetically attractable are well known in the art and include, for example, particle density, particle mass, particle composition, particle buoyancy, and the size of a magnet used to create the magnetic field. Preferred particles that are not sufficiently magnetically attracted by a magnetic field when not aggregated have a maximum dimension of about 20 nm.

A probe may be attached to a detection group particle or solid matrix using covalent linkages, chelation, ionic interactions, through the use of a binding partner set (i.e., a ligand and its hapten) using any of a variety of methods that are well known in the art.

Probes, bridging molecules and optional linking molecules are nucleic acids (RNA or DNA), preferably oligonucleotides, containing base sequences that permit specific hybridization under defined conditions. These molecules may contain additional chemical groups, so long as these groups do not prevent hybridization under the defined conditions. These nucleic acids may contain conventional bases (adenine, guanine, cytosine, thymine, and uracil) which allow hydrogen bonding to a complementary base and may contain nonconventional bases (e.g., inosine) that can bond with different nucleotide bases. Moreover, these nucleic acids may contain well known derivatives or modified purine or pyrimidine bases, such as, for example, N^4-methyl deoxygaunosine, deaza- or aza- purines and pyrimidines, pyrimidine bases having substituent groups at the 5 or 6 position, and purine bases having an altered or a replacement substituent at the 2, 6 or 8 positions, exemplified by 2-amino-6-methylaminopurine, O^6-methylguanine, 4-thio-pyrimidines, 4-amino-pyrimidines, 4-dimethylhydrazine-pyrimidines, O^4-alkyl-pyrimidines, (e.g., see Cook, PCT Publication No. WO 93/13121; *The Glen Report, vol. 1*, 1993). Some residues in the nucleic acid may be "abasic", i.e., a

backbone structure without an attached purine or pyrimidine. The backbone may be a standard sugar-phosphodiester type backbone and/or a peptide nucleic acid backbone (e.g., as described by Hydig-Hielsen and Godskesen, PCT Publication No. WO 95/32305). Examples of other types of non-nucleotide linkages are well known (as described by Arnold *et al.*, PCT Publication No. WO 89/02439, and U.S. Pat. No. 5,585,481). Synthetic nucleic acids of these types can be produced using standard techniques well known in the art (e.g., Eckstein, F., *Oligonucleotides and Analogues, A Practical Approach*, chapters 1-5, 1991; Caruthers *et al.*, *Methods In Enzymology* vol. 154 p. 287 (1987); Bhatt, U.S. Pat. No. 5,252,723; and Klem *et al.*, WO 92/07864; Cook, PCT Publication No. WO 93/13121; Miller *et al.*, PCT Publication No. WO 94/15619; McGee *et al.*, PCT Publication No. W0 94/02051; Cook *et al.*, PCT Publication No. WO 94/19023; and Hydig-Hielsen and Godskesen, PCT Publication No. WO 95/32305). Oligonucleotide probes and linking molecules that are not intended to function as primers may optionally include a blocking group (e.g., alkyl groups, non-nucleotide linkers, alkane-diol, dideoxynucleotide residues, and cordycepin) at the 3' end so that the 3' OH group cannot accept a nucleoside triphosphate in a polymerization reaction.

Preferably, the first and second probes independently comprise (a) a backbone made up of one or more sugar-phosphodiester type groups and/or one or more peptide nucleic acid groups, and (b) independently selected base sequences comprising adenine, guanine, cytosine, thymine, uracil or analogs thereof, joined to the backbone. Even more preferably, the first and second probes substantially comprise components independently selected from the group consisting of: deoxyribonucleotide, ribonucleotide, 2'-methoxy substituted ribonucleotide, and 2'-halo substituted ribonucleotide. "Substantially comprise" indicates that one or more of the component(s) makes up at least 70%, more preferably, at least 80%, more preferably at least 90%, and most preferably 100% of the probe.

Samples may be from any source that contains a nucleic acid that is itself detectable or can be amplified to produce detectable nucleic acid. For example, a sample may be a biological specimen such as blood, other tissue or a bodily fluid such as lymph or urine. A sample may be a plant material or foodstuff that contains nucleic acid, either normally or resulting from contamination by a biological agent (e.g., a bacterium, fungus, or virus). Similarly, a sample may be an environmental sample such as water that is being tested for the presence of specific contaminants such as fecal bacteria or virus. The source of the sample is immaterial to the present methods, so long as it does not contain materials that interfere with nucleic acid hybridization to produce hybridization complexes or nucleic amplification, if amplification is required for formation of detectable hybridization complexes.

The examples provided below illustrate some embodiments of the present invention. These examples are not intended to limit the claimed invention, but rather illustrate methodology useful for practicing the present invention.

Example 1: Covalent Joining of a Detection Group to a Probe

This protocol was used to couple probes having SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4 to magnetic particles and red colored latex particles. Each oligonucleotide was coupled to the particles independently of the other oligonucleotides to produce probe-specific particles. Superparamagnetic particles of 1 μm (SeraMag™, Seradyn, Indianapolis, IN) or colored (e.g., white, red, blue or black) latex particles of 0.4 μm were washed twice with 0.1 M imidazole, pH 6, at room temperature. The SeraMag™ particles were separated from the wash solution by placing samples in a magnetic rack; the colored latex particles were separated by centrifugation at 10,000 rpm for 3 to 5 min.

An oligonucleotide with a primary amine-terminated linker (as described in detail in U.S. Pat. No. 5,585,481) was combined with the particles in 0.1 M imidazole, using an oligonucleotide concentration of 17.3 nanomoles/mg of particles, and a 4% suspension of particles. In this mixture, 3-(3'-Dimethylaminopropyl)-1-ethyl carbodiimide was dissolved to produce a final concentration of 0.2 M, then the mixture was incubated at 65°C for 2 hr with constant agitation to couple the oligonucleotides to the particles.

The oligonucleotide-coupled particles were then washed at a concentration of 1% solids by suspending the particles in a solution, separating the particles and removing the wash solution in the following sequence: two times with 0.1 M imidazole, pH 6, at room temperature; two times with 0.1 M potassium carbonate at room temperature; three times with 0.1 M potassium carbonate at 65°C; and two times with water at room temperature. The particles were separated as described above and stored as 1% solids.

Example 2: Visual Detection of a Target Sequence by Solution Clearing

This example illustrates the detection of a hybridization complex formed with a first probe joined to an opaque detection group and a second probe joined to a paramagnetic detection group. A heterogeneous preparation of polydeoxyadenosine (Poly-dA; from SIGMA®, St. Louis, MO) was used as the target sequence. The presence of the Poly-dA target oligonucleotide was detected by a clearing of the solution without the aid of instrumentation.

Mixtures of superparamagnetic particles (65 μg) joined to probe consisting of SEQ ID NO:2, substantially as described in Example 1, 40 μg of colored 0.4 μm latex beads (white, red or black) joined to probe having SEQ ID NO:2, produced as described in Example 1, and 100 μl of General

Hybridization Buffer (50 mM lithium succinate, pH 4.7, 0.6 M LiCl, 1% lithium lauryl sulfate, 10 mM EDTA, 10 mM EGTA, 7.5 mM aldrithiol and 1.5% ethanol) were made in multiple tubes. To this mixture was added either 1 μg of Poly A (experimental tubes) or an equal volume of water (control tubes), and the mixture was allowed to stand at room temperature for 30 min, when the tubes were placed in magnetic racks to provide a magnetic field to the outside of the tubes. The magnetic field for these tests was applied to a side of the tube, below the top of the solution (within about 1 cm of the tube bottom), and changes were observed in the solution within about 5 min.

Those mixtures which received no Poly A remained the same color as the color of the latex particles added (e.g., if red particles were used, the solution was red). Those mixtures which received the Poly A target nucleic acid became clear indicating a target-dependent interaction between the particles which were then drawn out of solution by application of the magnetic field.

Example 3: Detecting Amplified Nucleic Acid

This example illustrates visual detection of amplified nucleic acid. Here, the amplified nucleic acid was first generated and then detected, although amplification and detection steps could be carried out simultaneously.

Amplification was performed in a solution composed of 5 μl each of primers having SEQ ID NO:5 and SEQ ID NO:6 (3 picomoles/μl), 25 μl of Amplification Reagent (160 mM Tris, pH 7.5, 20% polyvinylpyrrolidone, 92 mM $MgCl_2$, 92 mM KCl, 16 mM of each of ATP, CTP and UTP, 20 mM GTP, 6 mM of each of dGTP, dCTP, dATP and dTTP), 5 μl of a target rRNA (1 fg/μl) and 35 μl water, with a 200 μl silicone oil overlay, which was heated to 95°C for 15 min. The solution was then incubated at 42°C for 10 min, and 25 μl of Enzyme Reagent (140 mM Tris, pH 8, 70 mM KCl, 15% Tween™40, 20% glycerol, 1 mM each of EDTA and EGTA, 150 mM NaCl, 2500 units Moloney murine leukemia virus (MMLV) reverse transcriptase (one unit polymerizes 5.75 fmoles cDNA in 15 min), 2000 units T7 RNA polymerase (one units produces 5 fmole RNA in 20 min)) was added and the mixture incubated 60 min more at 42°C. Target rRNA was obtained from a *Mycobacterium complex* organism, as described in Hogan *et al.*, U.S. Pat. No. 5,547,842.

The amplification reaction was quenched with 100 μl of Particle Binding Buffer (190 mM HEPES, pH 7.5, 2% lithium lauryl sulfate, 806 mM lithium chloride). Serial dilutions of the produced amplification products ("amplicon") were then prepared using Particle Binding Buffer as the diluent.

The hybridization mixture included 56 μg of red latex beads having attached SEQ ID NO:4 probes joined at their 5' ends to the beads through a non-nucleotide linker (as described in Example 1), 94 μg of superparamagnetic particles having attached SEQ ID NO:3 probes joined at their 5' ends to the particles through a non-nucleotide linker (as described inExample 1). The mixtures also

included 100 μl of Particle Binding buffer, to which was added 10 μl of the diluted amplicon (5,000, 500, 50 or 5 fmoles), and made to a total volume of 200 μl with water. The mixtures were allowed to stand 12 hr at room temperature after which the tubes were placed in a magnetic rack and observed.

Using visual detection, hybridization mixtures containing 5,000 fmoles of amplicon appeared clear. A diminution of color was noted for mixtures containing 500 fmoles of amplicon, but no difference could be distinguished between mixtures containing 50 or 5 fmoles of amplicon and the control mixture containing no amplicon.

Example 4: Amplification of Target Nucleic Acid in the Presence of Detection Groups

This example shows that a target nucleic acid (rRNA isolated from *M. tuberculosis*) can be amplified in the presence of detection groups with attached probes, specifically 0.4 μm latex particles and SeraMag™ magnetic particles, as described in Example 3. Amplification of the rRNA target was performed using a transcription associated amplification procedure (as described in detail in U.S. Pat. Nos. 5,480,784 and 5,854,516) and amplified product was detected using a specific probe labeled with a chemiluminescent label and detected in a homogeneous detection procedure (as described in detail in U.S. Pat. Nos. 5,547,842, and 5,639,604). The amplification procedure is substantially as described in Example 3; the signal detected in the chemiluminescent detection procedure is reported as "Relative Light Units" ("RLU"; an arbitrary measurement of light produced) which were detected using a luminometer (e.g., LEADER™ 450i luminometer; Gen-Probe Inc., San Diego, CA). In all amplification and detection mixtures, the same amount of target (0, 5 or 50 fg) was added. To half of the samples, particles (20 μg of red latex beads and 50 μg of SeraMag™ particles) were added before amplification was initiated. All combinations were tested in triplicate and the amplification reported in Table 1 is the average (mean) of the RLU detected for each combination.

Table 1: Amount of Amplification Products Detected (Mean RLU)

Particles Present	No Target	5 fg Target	50 fg Target
Yes	2255	79,265	158,254
No	2125	1,716,322	2,869,362

The results in Table 1 show that amplification of target nucleic acid occurred in reactions mixtures that included detection particles with attached probes. The relative numbers of RLU detected are probably not directly proportional to the amount of amplicon produced because the particles absorb some of the light. Detectable amplified nucleic acids were produced in the

presence of detection groups and attached probes.

This example illustrates another advantage of this method: that the assay can be practiced in essentially a closed system once the sample has been added because amplification can proceed in the presence of detection probes. Therefore, potential contamination resulting from multiple additions of reactants to the assay is avoided, making the method particularly suitable for field testing.

Example 5: Quantitative Detection of Agglutination of Hybridization Complexes

This example shows that optical detection of hybridization complexes formed under conditions that mimic transcription-associated amplification can quantitatively detect different amounts of agglutination. For this experiment two sets of probes bound to detection groups were used, with all of the probes attached to the particles at the 5' end substantially as described in Example 1. The first set of probes contained a spacer sequence consisting of a poly(dT)$_{30}$ sequence joined to the probe and a target-complementary sequence 18 residues (Probe 1; SEQ IN NO:4) or 20 residues (Probe 2; SEQ ID NO:3) joined covalently to the 3' end of the poly(dT)$_{30}$ sequence. Probe 1 was joined to superparamagnetic particles (SeraMag™ beads) and Probe 2 was joined to red 0.4 μm latex beads, substantially as described in Examples 1 and 2. The second set of probes were attached to the same type of particles, where the attached sequences that were essentially head-to-tail dimers of the non-poly(dT) sequences of Probes 1 and 2. That is, Probe 3 was an oligomer of 38 residues (SEQ ID NO:7) consisting of a first copy of the non-poly(dT) sequence of Probe 1, two T residues, and a second copy of the non-poly(dT) sequence of Probe 1; Probe 4 was an oligomer of 40 residues (SEQ ID NO:8) consisting of a head-to-tail dimer of the 20-mer non-Poly(dT) residues of Probe 2, without any spacer sequence. The non-poly(dT) sequences of these probes are complementary to two different regions of the synthetic target sequence (a DNA oligomer of 133 residues, whose sequence is derived from a *M. tuberculosis* 23S rRNA sequence). Probe 3 was attached to superparamagnetic particles and Probe 4 was joined to red 0.4 μm latex beads, substantially as described in Examples 1 and 2.

Hybridization reactions were tested in duplicate and each contained one set of probe particles using a combination of 20 μg of latex bead-attached probes and 30 μg of superparamagnetic particle-attached probes. The 100 μl reaction mixtures contained 0, 1 or 10 pmoles of synthetic target, 25 μl of Amplification Reagent (see Example 3), and 25 μl of Enzyme Reagent (see Example 3), prepared without the enzymes. The mixtures were incubated 15 min at 42°C and then the hybridization complexes were separated by applying a magnetic field for at least 5 min as described in Example 2. Then, the solution was removed, diluted 10-fold, and the

absorbance at 500 nm (A_{500}) was measured using standard spectroscopy methods. Table 2 shows the average results (Mean A_{500}) of these tests.

Table 2

Probe Combination	Target (pmoles)	Mean A_{500}
1 and 2	0	0.441
1 and 2	1	0.095
1 and 2	10	0.080
3 and 4	0	0.307
3 and 4	1	0.118
3 and 4	10	0.030

As can be seen from the results presented in Table 2, absorbance at 500 nm was relatively high for all tubes that did not contain the target nucleic acid, and decreased significantly in mixtures that contained 1 or 10 pmoles of target. These results show that hybridization complexes formed in mixtures containing target and the magnetic field then removed the complexes resulting in an optically detectable signal (decrease in absorbance) that was quantitative.

Other embodiments are within the following claims. Thus, while several embodiments have been shown and described, various modifications may be made, without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. A method of detecting a target nucleic acid in a sample comprising the steps of:

providing a sample containing multiple copies of a target sequence;

mixing the multiple copies of the target sequence with a probe reagent comprising a first probe attached to a first detection group, and a second probe attached to a second detection group, thereby producing a hybridization mixture;

incubating the hybridization mixture under conditions that favor hybridization of at least a portion of the first probe with a bridging molecule, and favor hybridization of at least a portion of the second probe with the bridging molecule, thereby producing a hybridization complex comprising the first probe and its attached first detection group, the second probe and its attached second detection group, and the bridging molecule; and

optically detecting formation of the hybridization complex, thereby indicating that the target nucleic acid is present in the sample.

2. The method of claim 1, wherein the multiple copies of the target sequence are present in the sample without further treatment of the sample.

3. The method of claim 1, wherein the multiple copies of the target sequence are produced by amplification of a target nucleic acid present in the sample.

4. The method of claim 3, wherein the multiple copies are produced by transcription-associated amplification of nucleic acid.

5. The method of claim 1, wherein the bridging molecule is a target sequence present in the sample, an amplification product of a target nucleic acid present in the sample, a nucleic acid sequence capable of hybridizing to the amplification product of a target nucleic acid present in the sample, or a combination thereof.

6. The method of claim 1, wherein the mixing and incubating steps are performed under essentially constant reaction conditions.

7. The method of claim 1, wherein:

the bridging molecule is an amplified nucleic acid,

the first probe hybridizes, directly or indirectly, to a first amplified nucleic acid sequence; and

the second probe hybridizes, directly or indirectly, to a second amplified nucleic acid sequence, thereby producing a hybridization complex comprising the first probe, the second probe, and the amplified nucleic acid.

8. The method of claim 1, wherein the mixing step uses the first detection group that is an

opaque particle and the second detection group that is a magnetically attractable particle, such that the hybridization complex formed in the incubating step is attractable by a magnetic field, thereby localizing the hybridization complex by application of a magnetic field during the detecting step.

9. The method of claim 1, wherein the first detection group and the second detection group used in the mixing step are made of acrylamide, and the incubating step produces a polyacrylamide gel phase that is optically detectable in the detecting step.

10. The method of claim 1, wherein the detecting step uses visual detection of a change in the hybridization mixture without aid of instrumentation.

11. The method of claim 1, wherein in the mixing step:

the first probe is an oligonucleotide having the sequence of SEQ ID NO:4 or SEQ ID NO:7, wherein the first probe is joined to an opaque particle using a 5' end or 3' end of the sequence,

the second probe is an oligonucleotide having the sequence of SEQ ID NO:3 or SEQ ID NO:8, wherein the second probe is joined to a magnetically attractable particle using a 5' end or 3' end of the sequence, and

the target sequence is characteristic of a *Mycobacterium complex* organism.

12. The method of claim 1, wherein the first detection group and the second detection group used in the mixing step have magnetic properties that are insufficient to make individual detection groups attractable by a magnetic field,

and wherein the incubating step produces a hybridization complex that is magnetically attractable by the magnetic field in the detecting step.

13. The method of claim 1, wherein the first probe is covalently joined to the first detection group, and the second probe is covalently joined to the second detection group.

14. The method of claim 1, wherein the mixing step further comprises adding at least one linking molecule to the hybridization mixture, and the incubating step produces a hybridization complex that further comprises at least one linking molecule.

15. A method of detecting a target nucleic acid in a sample comprising the steps of:

providing a sample containing multiple copies of a target sequence;

mixing the multiple copies of the target nucleic acid sequence with a probe reagent comprising a first probe attached to a first detection group, and a second probe attached to a second detection group, and a bridging molecule capable of hybridizing to both the first and second probes, thereby producing a hybridization mixture;

incubating the hybridization mixture under conditions that favor hybridization of the first and second probes with the bridging molecule to produce a hybridization complex comprising the first

probe and its attached first detection group linked to the second probe and its attached second detection group by hybridization with the bridging molecule; and

optically detecting inhibition of formation of the hybridization complex by competition hybridization between the target sequence and at least one of the first probe, the second probe or the bridging molecule.

16. A probe reagent composition, comprising:

a first probe attached to a first detection group; and

a second probe attached to a second detection group,

wherein each of the probes comprises

a backbone made up of one or more sugar-phosphodiester type groups, one or more peptide nucleic acid groups, or a combination thereof, and

independently selected sequences of bases comprising adenine, guanine, cytosine, thymine, uracil or analogs thereof, joined to the backbone;

and wherein each of the detection groups comprises a composition that, when aggregated, is capable of being optically detected as a change in characteristics of a solution containing the detection groups in a non-aggregated form.

17. The probe reagent composition of claim 16, further comprising at least one linking molecule, wherein at least one linking molecule contains a base sequence capable of hybridizing to a selected sequence of bases contained in at least one probe.

18. The probe reagent composition of claim 16, wherein the first probe has the sequence of SEQ ID NO:3 or SEQ ID NO:8, and the second probe has the sequence of SEQ ID NO:4 or SEQ ID NO:7.

FIG. 1A

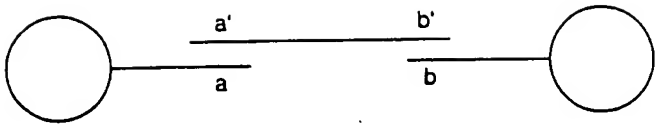


FIG. 1B

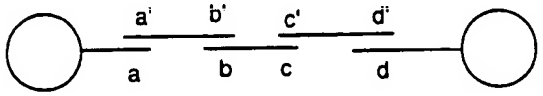


FIG. 1C

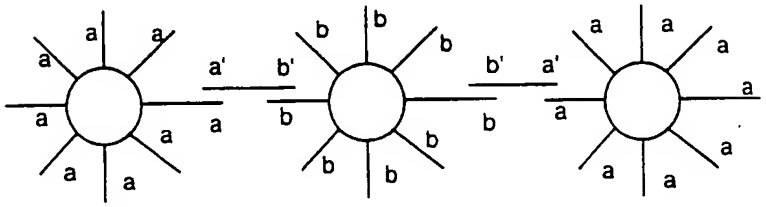
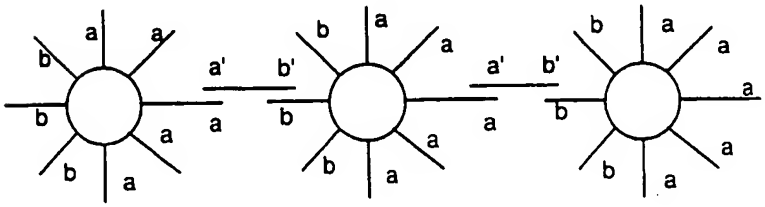


FIG. 1D



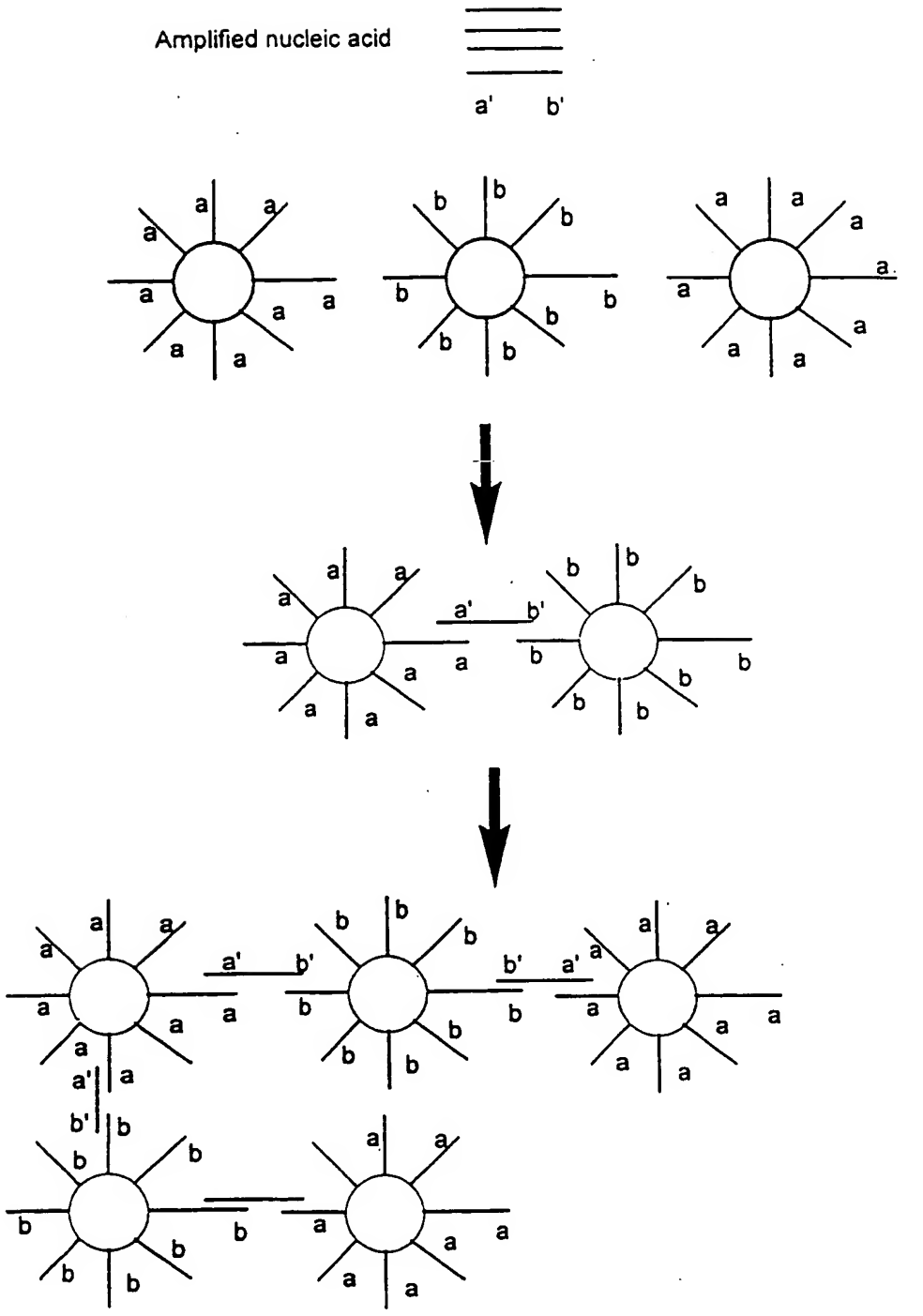


FIG. 2

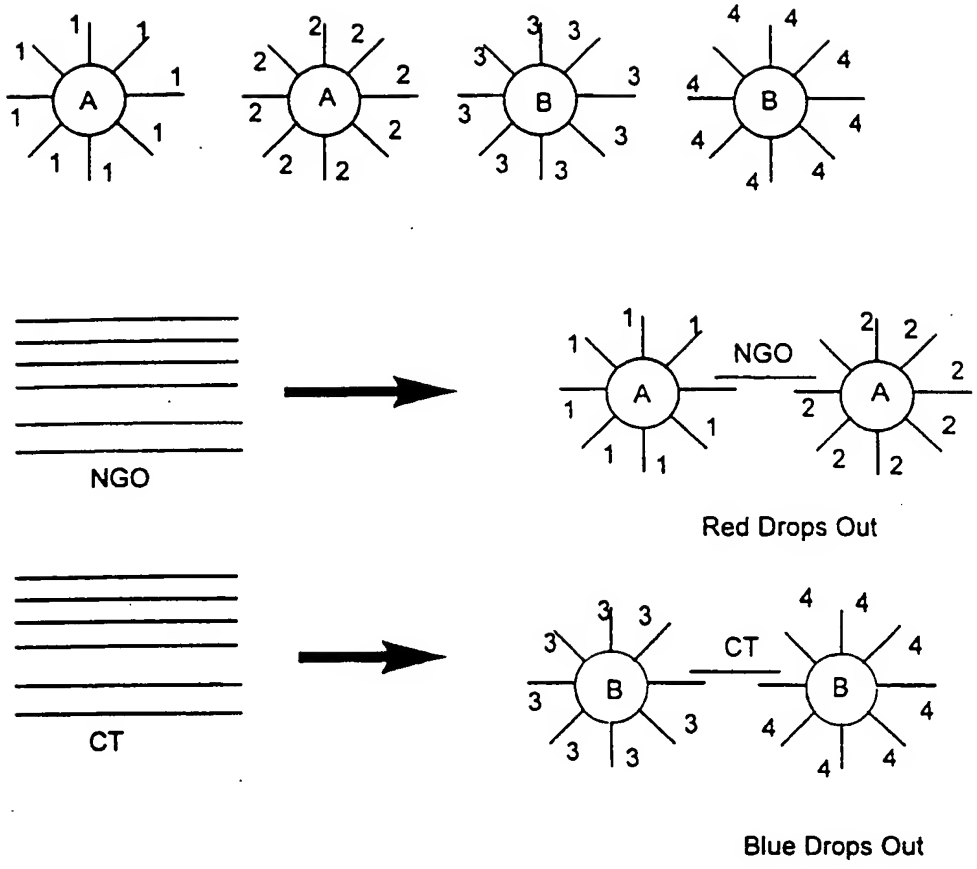


FIG.3

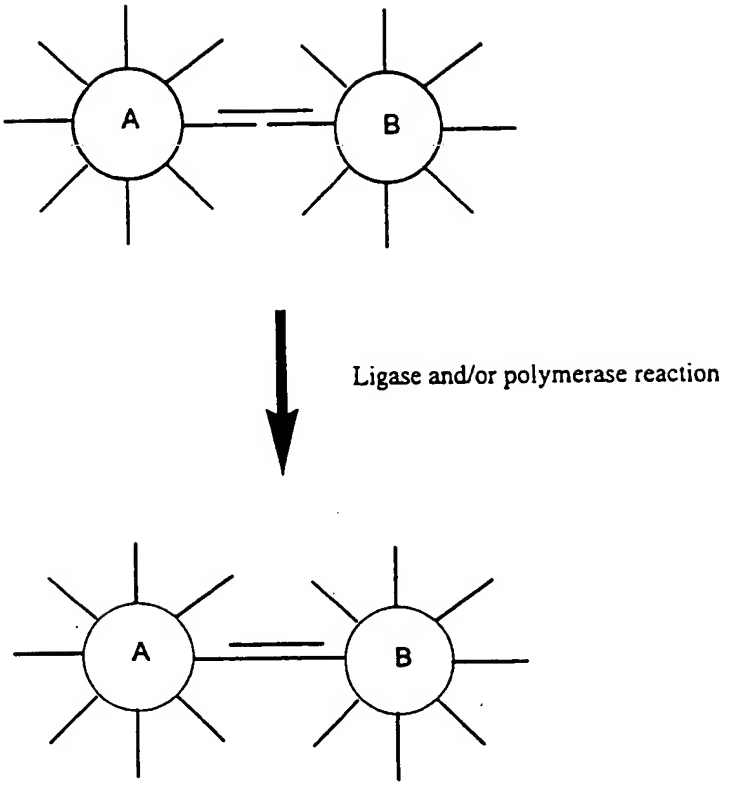


FIG. 4

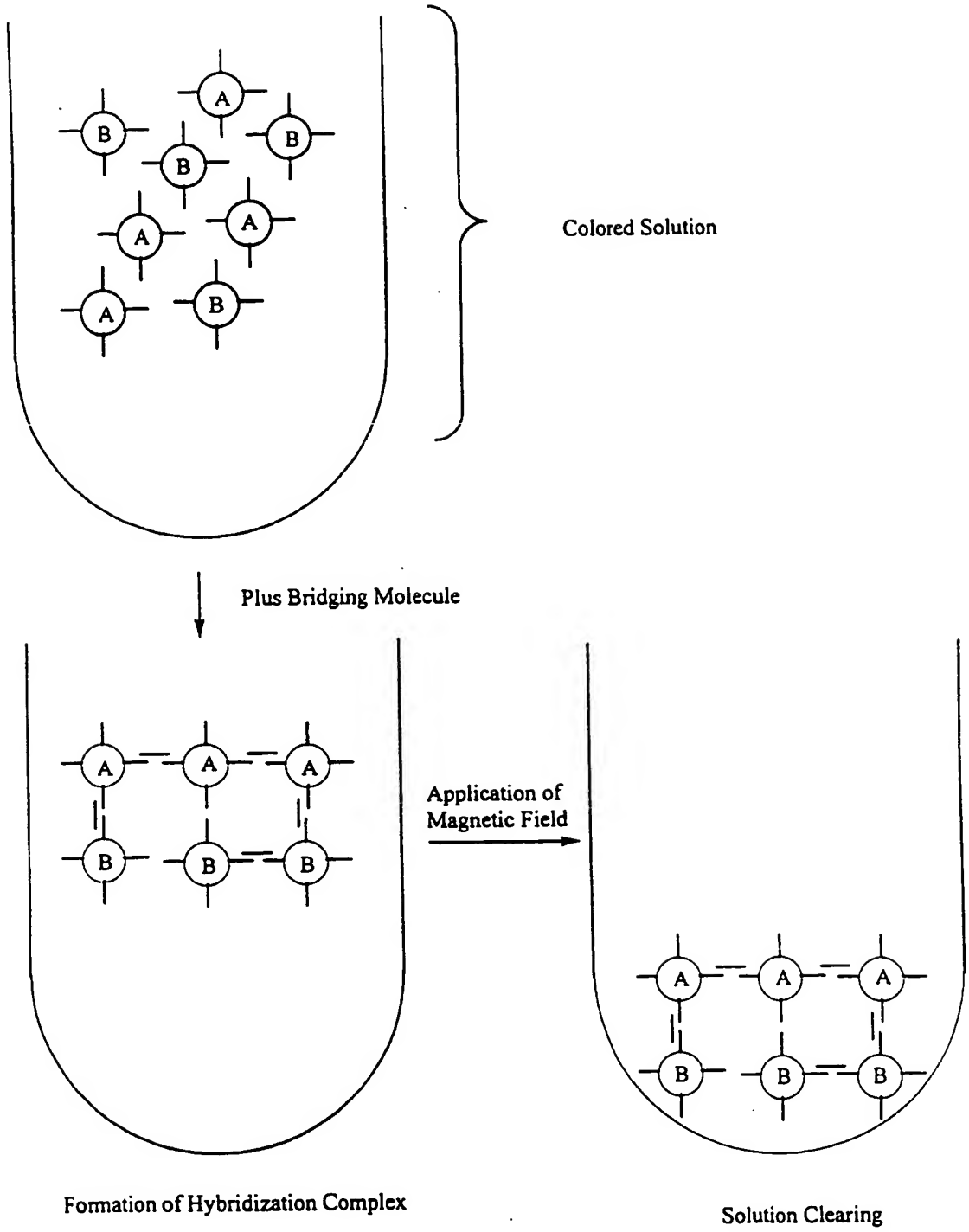

A
B
B
B
A
A
A
B
Colored Solution
Plus Bridging Molecule
A
A
A
B
B
B
Application of
Magnetic Field
A
A
A
B
B
B
Formation of Hybridization Complex
Solution Clearing

FIG. 5

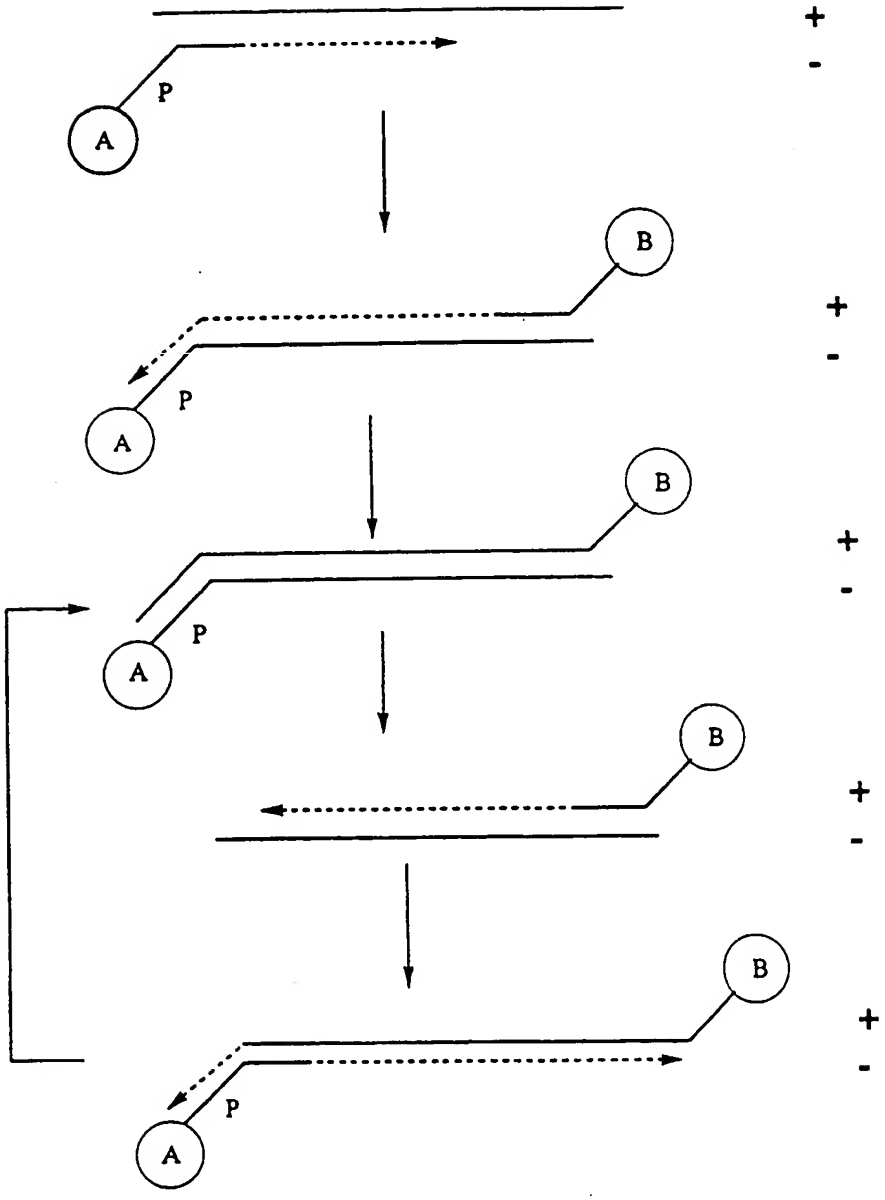
FIG. 6A

FIG. 6B

FIG. 6C

FIG. 6D

FIG. 6E



SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Gen-Probe Incorporated

(ii) TITLE OF INVENTION: METHODS OF NUCLEIC ACID DETECTION

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:

(A)	ADDRESSEE:	Gen-Probe Incorporated
(B)	STREET:	10210 Genetic Center Dr.
(C)	CITY:	San Diego
(D)	STATE:	California
(E)	COUNTRY:	U.S.A.
(F)	ZIP:	92121-4362

(v) COMPUTER READABLE FORM:

(A)	MEDIUM TYPE:	3.5" Diskette
(B)	COMPUTER:	IBM Compatible
(C)	OPERATING SYSTEM:	ASCII DOS Text
(D)	SOFTWARE:	FastSEQ for Windows 2.0

(vi) CURRENT APPLICATION DATA:

(A)	APPLICATION NUMBER:	To Be Assigned
(B)	FILING DATE:	Herewith
(C)	CLASSIFICATION:	

(vii) PRIOR APPLICATION DATA:

(A)	APPLICATION NUMBER:	US 60/063,969
(B)	FILING DATE:	31 October 1997

(viii) ATTORNEY/AGENT INFORMATION:

(A)	NAME:	Christine Gritzmacher
(B)	REGISTRATION NUMBER:	40,627
(C)	REFERENCE/DOCKET NUMBER:	GP093-PCT

(ix) TELECOMMUNICATION INFORMATION:

(A)	TELEPHONE:	(619) 410-8926

(B) TELEFAX: (619) 410-8928
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTTTTTTTTT TTTT 14

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT 30

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT CGGCTGAGAG GCAGTCAGAA 50

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT GGTAGGGGTT GTGTGTGC 48

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	24 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGCGGAACAG GCTAAACCGC ACGC 24

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	47 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AATTTAATAC GACTCACTAT AGGGAGACCA GGCCACTTCC GCTAACC 47

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	38 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGTAGGGGTT GTGTGTGCTT GGTAGGGGTT GTGTGTGC 38

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	40 base pairs
(B)	TYPE:	nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGGCTGAGAG GCAGTCAGAA CGGCTGAGAG GCAGTCAGAA 40

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/23267

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 90 02205 A (ANGENICS INC) 8 March 1990 see the whole document ---	1-10, 12-17 11,18
X Y	WO 97 23647 A (BEHRINGWERKE AG ;ULLMAN EDWIN F (US)) 3 July 1997 See page 7, line 33-43 and page 8, line 1-5; page 28, line 40-42 and page 29, line 1-13; page 33, line 1-3; Example 2. see the whole document ---	1-10, 12-17 11,18
X Y	WO 87 05334 A (ANGENICS INC) 11 September 1987 see the whole document --- -/--	1-7,10, 13-17 8,11,12, 18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1999	23/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	WO 94 03472 A (GEN PROBE INC) 17 February 1994 See Mycobacterium 23S rRNA detection helper probe #2. Oligonucleotide with 100% identity in 18 bp overlap with Seq.ID 4 and Seq.ID 7.	11,18
Y	DATABASE EMBL ID/AC: I21510, 14 October 1996 SHAH ET AL.: "OLIGONUCLEOTIDES COMPLEMENTARY TO MYCOBACTERIAL NUCLEIC ACIDS" XP002096127 Oligonucleotide with 100% identity in 20 bp overlap with Seq.ID 3 and Seq.ID 8 see abstract	11,18
Y	DATABASE EMBL ID/AC: A42710/ Seq. ID 42 from WO9503412, 6 March 1997 MABILAT AND CHRISTEN: "NUCLEOTIDE FRAGMENT OF MYCOBACTERIAL RIBOSOMAL RNA 23S, PROBES AND PRIMERS DERIVED THEREFROM, REAGENT AND METHOD FOR DETECTING SAID FRAGMENT" XP002096128 Oligonucleotide with 100% identity in 20 bp overlap with Seq.ID 3 and Seq.ID 8 see abstract	11,18
Y	US 4 628 037 A (JOSEPHSON LEE ET AL) 9 December 1986 see the whole document	8,12
A	SAFARJK ET AL.: "The application of magnetic separations in applied microbiology; equipment and procedures, applications in immuno-magnetic separation, and detection procedures, and use in waste-water treatment, immobilization, etc." J.APPL.BACTERIOL., vol. 78, no. 6, 1995, pages 575-585, XP002096125 see the whole document	1-18
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INTERNATIONAL SEARCH REPORT

Interi nal Application No
PCT/US 98/23267

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
P,X	BAINS: "SIMPLE DNA PROBE ASSAYS BASED ON PARTICLE AGGLUTINATION" CLIN.CHEM., vol. 44, no. 4, April 1998, pages 876-878, XP002096126 cited in the application see the whole document	1-7,10, 13-17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/US 98/23267

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